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Research Article

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Comparison of Near-infrared Spectroscopy with other options for total carotenoids content phenotyping in fresh cassava roots

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ABSTRACT

This study compared the relationship of different phenotyping methods including iCheck™ CAROTENE (iCheck), Chromameter, colour chart and visible/near-infrared spectroscopy (Vis/NIRS) used in quantifying total carotenoids content (TCC) in fresh cassava roots. Using a total of 194 cassava clones harvested from the International Institute of Tropical Agriculture (IITA), Ibadan, we compared the repeatability precision, accuracy of measurement and correlations of these phenotyping methods. From the results, Vis/NIRS-analyzed TCC had high and positive correlations with Chromameter and Color chart (r = 0.91 and 0.71, respectively). On the other hand, the result revealed somewhat moderate correlation (r = 0.67) between Vis/NIRS and iCheck measurements. Vis/NIRS, iCheck and chromameter methods gave high and nearly equal heritability estimates (0.95, 0.98 and 0.98, respectively) illustrating high repeatability precision of these methods; an indication that they can be used for germplasm selection in the early stages of breeding. Conversely, with Bland-Altman plot at 95% confidence level, the accuracy of iCheck was not comparable with that of Vis/ NIRS. The information derived from this analysis directly contributes towards the genetic improvement of root quality traits in cassava and facilitates the sharing of data across cassava breeding consortium.

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INTRODUCTION

Cassava (*Manihot esculenta*, Crantz) continues to make a significant impact in the lives of over 800 million people worldwide where it serves as a staple food, feed for animals, fuel and source of income especially for the low income population[1, 2, 3]. It is estimated that two out of every five people in Africa consume cassava on daily basis [4]. This popularity could be attributed to the diversity of consumption options of the roots as well as leaves that are consumed as vegetable in many regions of the world [5]. Cassava has a significant leverage over legumes and cereals as a staple largely because the crop is drought tolerant, produces well in soils of low fertility and retains the ability to store for longer periods underground [6]. Taken together, these attributes make cassava popular among resource-poor farmers, particularly in regions where effects of climate change is becoming apparent [7].

Inherently, cassava roots are major starch sink and thus a source of carbohydrates. Consequently, communities that heavily depend on cassava roots for food tend to be highly vulnerable to vitamin A deficiency [8]. Vitamin A is an important micronutrient for the normal functioning of the eyes, immune systems, growth and development, maintenance of epithelial cellular integrity and reproduction [9]. Indeed, reports [10] indicate that Africa has the highest proportion (2%) of pre-school age children affected by night blindness, a value that is ten times higher than estimates from the West pacific (0.2%).

On a positive note, it has been found that cassava roots with high levels of carotenoids are a good source of vitamin A [11]. This is an exciting opportunity that can be fully exploited for the benefit of mankind. It is these nutritional benefits that are driving the current global bio-fortification efforts to develop cassava varieties enriched with pro-vitamin A carotenoids.

Adequate genetic variations across breeding programs create an opportunity to improve on these traits in meeting up with the bio-fortification target for many food crops including cassava [12]. The International Centre for Tropical Agriculture (CIAT) has reported TCC as high as 29 μg/g [13,14]. Relatedly, the International Institute for Tropical Agriculture (IITA) developed cassava clones with TCC as high as 10 μg/g, of which five have been officially released and are under production in Nigeria [15].

Indeed, deploying bio-fortified cassava would be a feasible means of reaching malnourished rural populations who have limited access to diverse diets, supplements and commercially fortified foods [16]. However, screening large breeding populations to quantify outstanding clones for official release is a major limitation to such breeding initiatives aiming at fast-tracking micronutrient increments in cassava [17]. In particular, protocols used to extract and quantify analytes from roots are tedious, expensive and slow for routine analyses of thousands of samples. High Performance Liquid Chromatography (HPLC) is a reliable method for quantification of pro-vitamin A carotenoids [18,19], but it can only be used to analyze a limited number (<10) of cassava samples per day [20]. This becomes a major bottleneck and has resulted in many interventions especially within the consortium of cassava breeding programs in Africa and South America. Some of the reported options for TCC quantification besides HPLC include the use of iCheck [21], Chromameter [22], spectrophotometer [21] and recently, the use of visible/near-infrared spectroscopy [14,22] that is now available in a portable format with flexible options for field based analyses [23]. Vis/NIRS has been demonstrated to be comparatively accurate, reliable, rapid and inexpensive for quantifying levels of pro-vitamin A carotenoids in cassava [24,20].

As more efforts are being put in place by many breeding programs in overcoming the challenges of developing a high throughput phenotyping for the quantification of key traits in cassava, we anticipate an increase in the development or the improvement of quick and low resource phenotyping options for existing and novel traits in cassava. However, considering the need to achieve a common goal and in so many cases facilitate the sharing of data from different breeding programs [25, 26], there is need to understand the correlation between the different phenotyping options available among breeding programs. This is important especially among programs with common goals which is currently demonstrated within the Next Generation Cassava breeding consortium [27]. The major goal of this study was to evaluate the relationship between different phenotyping methods available at different breeding programs for TCC quantification on fresh cassava roots which is relevant for future investments in phenotyping and the sharing of data.

MATERIALS AND METHODS

Study Population

A diverse collection of cassava clones, commonly referred to as genetic gain population developed over years at IITA was used for this study. This population has been used in a number of studies including dissection of genetic architecture for cassava mosaic disease[25] and more recently in studies examining the prospects of genomic selection in cassava[26]. The genetic gain population was planted on May 2015 at Ubiaja, a field station for IITA in Nigeria, located at an altitude of 221m, 6^0 38'57.59"N (Latitude) and 6^0 23'30.35"E (Longitude). The mean annual rainfall ranges between 1,800 mm to 2,000 mm. For this study, a subset of the genetic gain population involving 194 clones with varying levels of root TCC were selected using a simple random sampling technique.

Sample Preparation

Three plants were harvested from a plot of ten plants and three roots per plot were used for analyses. Sampled roots were transported to IITA's laboratories at Ibadan within six hours after harvest and stored for a maximum of one day in the freezer at 0°C. Prior to analyses, the root samples were peeled, washed and labeled appropriately. Special care was taken during sample preparation to avoid direct exposure to sunlight by covering the lights in the laboratory with filters. Each of the three roots was cut longitudinally into half, thereafter the two halves were again cut longitudinally to generate quarters. Two opposite quarters were selected from each root (making six quarters from three roots) and chopped into extremely tiny cubes of approximately less than 0.2 cm^3 and mixed together. After thorough mixing, 150g of cassava roots was packed in a transparent zip polythene bag as a uniform and representative sample for carotenoids analysis.

Spectra Collection and Analysis

Spectra data were collected from the 194 clones of the IITA's genetic gain population using a portable Vis/NIRS device (QualitySpec Trek: S-10016). Chopped root samples were fed into quartz sampling cups and spectra were collected by placing the cups against the window of the portable Vis/NIRS device. Three replications per sample were collected by taking readings from three cups of the homogenized sample.

TCC was analyzed using the Vis/NIRS calibration model developed at National Root Crops Research Institute (NRCRI), Umudike on intact root samples [23]. The calibration model was developed using 113 calibration samples with references derived using standard laboratory procedures for TCC at NRCRI carotenoids laboratory [28]. The calibration model was developed using Win-ISI 4.5 software (Infrasoft International and FOSS, Hillerod, Denmark). Spectra pre-treatment was carried out using the standard normal variate and detrend (SNV-D) on five data points and smoothed using Savitzky–Golay polynomial smoothing on five data points. Modified partial least square (MPLS) chemometric algorithm was used to develop prediction model on log 1/*R* of spectral wavelength. Outliers were identified using the student (*t*) test based on the standardized residuals with a cut-off of 2.5. Outliers were eliminated using two passes. The accuracy of the final MPL model was evaluated in terms coefficient of determination (R^2) of the model and the standard error of cross-validation (SECV) [23].

TCC Quantification using iCheck

About 5g of the chopped and homogenized root samples were ground using a mortar and pestle to form a fine paste. Sample

grinding was aided by adding 20ml of distilled water and the resulting solution transferred into a 50ml calibrated falcon tube. Contents of falcon tube were thoroughly shaken to get homogeneous slurry. Immediately after vigorous shaking, 0.4ml of slurry was injected into the reagent vial and vigorously shaken for 10 seconds. The vial was placed on a solid surface for approximately 5 minutes and then again shaken for 10 seconds. At this point, the vial was left to stand until two solution phases appeared inside the vial: a clear upper phase and a turbid lower phase. Absorbance of the vial content (the upper solution phase) was measured using the iCheck device, and TCC calculated as:

$$TCC(\mu g / g) = \frac{TS_v}{S_w} \times A$$

Where TSv = total sample volume of slurry dispensed to the falcon tube (in this case 25ml), S_w = weight of a sample (5g) and A = absorbance of the reagent vial content displayed by iCheck device. Absorbance readings were taken for each sample in three replicates from three subsamples of homogenized root sample.

TCC Quantification using Chromameter

Chromameter (CR 400) works by using the *Lab* colour space that mathematically illustrates all perceivable colours in three dimensions; L^*, a^* and b^* where L^* represent lightness, a^* represent green-red and b^* represent blue-yellow colours. The values of L^*, a^*, and b^* are usually absolute. The value $L^* < 0$ represent the darkest black while $L^* = 100$ represents brightest white. The values $L^* = 0$, $a^* = 0$ and $b^* = 0$ represents neutral grey. The negative values of a* axis represent colour green while the positive values represents colour red. The negative values of b* axis represent colour blue while the positive values represents colour yellow. The limits of a^* and b^* axes are often in the range of ±100 or run from -128 to +127. For TCC measurement, positive values in b* axis are used. In this axis, as you move from 0 to +100, the yellowness increases. Therefore, the higher the TCC in cassava root, the higher the amount of positive b* axis values read by the chromameter. The chromameter (CR 400) device was used for TCC estimation in fresh cassava root sample [22].

TCC Evaluation by Color Chart

Root flesh colour was scored for all genotypes using visual inspection following the qualitative colour chart in the scale of 1-8 [11].

Data Analysis

We summarized the values derived from each phenotyping method in terms of coefficient of variation (CV), standard deviation (SD), average, maximum and minimum values. Bland-Altman plot generated using GraphPad Prism v7.01 software [29] was used to compare the agreement in terms of accuracy of NIRS and iCheck method [30]. Bias, the average of differences between predicted values by Vis/NIRS (y_i) and observed values by iCheck (x_i), was computed as:

$$Bias = \frac{1}{N}\sum_{i=1}^{N}(y_i - x_i)$$

Where N = number of samples.

Pearson correlation coefficients (r) amongst the generated datasets were calculated using R v3.0.2 software [31]. In addition, broad sense heritability (H^2) estimates associated with each measurement were computed to assess the repeatability precision of these methods. This was done by computing genotypic and phenotypic variances, and thereafter broad sense heritability [32].

RESULTS

Summary statistics for TCC as quantified by the four phenotyping methods are shown in Table 1. The average TCC (fresh weight basis) as recorded by the devices showed an average value of 3.79µg/g using iCheck, 3.24µg/g from Vis/NIRS, average b* axis value (colour intensity) of 26.66 using Chromameter and average color scale of 2.21 on a scale of 1-8. The maximum TCC value was 10.18µg/g from iCheck, 5.87µg/g from Vis/NIRS, a color scale of 4.00 using color chart and b* axis value of 39.62 using Chromameter. The coefficient of variation (CV) for TCC was highest (65.2%) and lowest (25.4%) using iCheck and Chromameter methods, respectively. Vis/NIRS and color chart had CV of 41.7% and 36.6% respectively. Similarly, the minimum values and standard deviations of TCC differed across methods (Table 1).

Also, there was significant ($p<0.001$) variability of TCC among the genotypes across iCheck, Vis/NIRS and Chromameter (Table 2). This variability and distribution was optimum for establishing the relationship and comparison of the phenotyping methods. Although the mean sums of squares were significantly different across methods, the heritability estimates were high. TCC data from Vis/NIRS, iCheck and Chromameter showed good repeatability precision; since all the three methods had

Table 1: Summary statistics of the data collected by iCheck, Vis/NIRS, Chromameter and Qualitative colour chart.

	iCheck (TCC µg/g)	Vis/NIRS (TCC µg/g)	Chroma (b* axis values)	Chart (TCC scale of 1-8)
Average	3.79	3.24	26.66	2.21
Max	10.18	5.87	39.62	4.00
Min	0.12	0.53	11.69	1.00
SD	2.47	1.35	6.78	0.81
CV %	65.2	41.7	25.4	36.6
Sample size	194	194	194	194

iCheck=iCheck, Chroma=Chromameter, Chart=Qualitative colour chart, TCC=Total carotenoids content, Vis/NIRS=Visible/Near-infrared spectroscopy, µg/g=Microgram per gram, Max=Highest value, Min=lowest value, SD=standard deviation, CV=coefficient of variation

high and almost equal heritability values (iCheck= 0.98, Vis/NIRS = 0.95, Chromameter = 0.98) irrespective of the disparity in their variance components (Table 2).

Overall, the correlations among the different methods for TCC phenotyping were positive and significantly high ($P<0.001$), with the highest values recorded between NIRS with Chromameter ($r = 0.91$). The correlation between Vis/NIRS and iCheck was the lowest correlation obtained from the study ($r = 0.67$). The correlation between the other three methods with color chart ranged from $r = 0.71$ with Vis/NIRS, $r = 0.77$ with Chromameter and $r = 0.84$ with iCheck (Table 3).

The Bland-Altman plot of difference between Vis/NIRS values and those of iCheck against their mean in a test of comparing their accuracy indicated incongruence of these methods on their TCC value at 95% confidence interval with a bias of 0.55 (Figure 1). This was seen as some data points laid outside the upper and lower limits.

Table 2: Variance components, mean squares and broad sense heritability of TCC using Vis/NIRS, iCheck and Chromameter

	Vis/NIRS	iCheck	Chromameter
Broad sense heritability	0.95	0.98	0.98
δ^2g	1.78	6.03	45.72
δ^2p	1.88	6.16	46.39
MS	5.43***	18.21***	137.83***

δ^2g = Genotypic variance, δ^2p= Phenotypic variance, iCheck= iCheck, Vis/NIRS=Visible/Near-infrared spectroscopy, MS=mean square, ***=Significance ($P<0.001$)

Table 3: Correlation coefficients amongst different methods for phenotyping TCC in cassava

	Chart	iCheck	Chroma	Vis/NIRS
Chart	1			
iCheck	0.84***	1		
Chroma	0.77***	0.72***	1	
NIRS	0.71***	0.67***	0.91***	1

***Significance ($P<0.001$), Chart = Qualitative colour chart using a scale of 1-8, iCheck = iCheck, Chroma = Chromameter, Vis/NIRS=Visible/Near-infrared spectroscopy

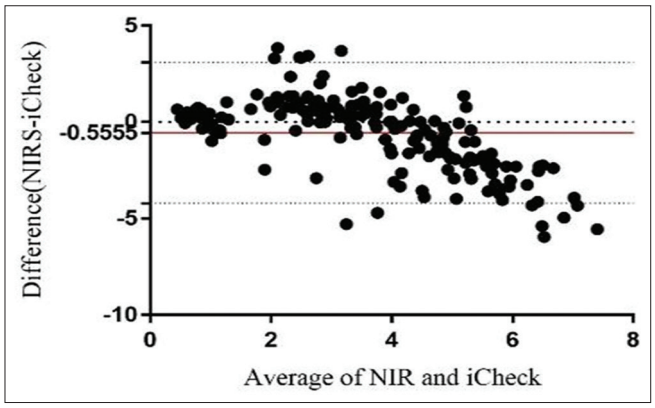

Figure 1: A Bland-Altman plot of the NIRS and iCheck differences against their mean values showing the upper and lower limits at 95% confidence interval

DISCUSSION

The objective of this study was to compare the relationship between different phenotyping methods available at different cassava breeding programs for TCC prediction on fresh cassava roots which is relevant for future investments in phenotyping and facilitates the sharing of data across cassava breeding consortium. This was achieved by assessing their correlation, agreement in terms of their accuracies and evaluating their repeatability precision towards the prediction of TCC in fresh cassava roots. As used in this study, precision is the closeness of values obtained through replicate measurements on the same sample. It represents a measure of dispersion of data around the mean value [33]. Indeed, precision is directly related with repeatability and also inversely associated with random errors. Thus for clarification, herein precision should not be taken as a synonym to accuracy. Output from this analysis directly contributes towards genetic improvement of root quality traits in cassava across cassava breeding consortium.

Precision was evaluated for Vis/NIR, iCheck and Chromameter by studying their repeatability. Based on repeatability precision analysis, all the three phenotyping methods appeared to be precise for quantifying TCC, given that they all had high and nearly equal heritability values. However, NIRS will offer the advantage of analyzing large number of samples over relatively shorter time than other methods, which is a critical requirement for breeding programs handling large populations [14,22].

Out of the four methods that were compared in this study, more emphasis was given to iCheck and Vis/NIRS because they phenotype TCC in the same units (µg/g) hence easy to compare on most statistical approaches. A comparison on accuracy of the phenotyping methods indicated a disparity between results from NIRS and iCheck. Notably, measurement of TCC in the same sample by the two methods showed a CV of 65.2% for data from iCheck, which was 23% higher than that of NIRS. It was expected that if the methods had similar accuracy, then they could have similar CV accounted only for the genetic differences among the genotypes. It is likely that the large portion of CV in iCheck data would be associated with high variations in measurements of extremely low and/or high values [34]. In the present study, maximum and minimum values of 10.17µg/g and 0.12µg/g, respectively, were recorded by iCheck. It is therefore probable that iCheck has a narrow limit of quantification compared with the other method, which essentially increases errors in quantifying extreme values, accounting for the large CV. Furthermore, results from Bland-Altman plot showed that at 95% confidence interval, some data points laid outside the minimum and maximum limits meaning that the two methods gave somewhat dissimilar values in measurement of TCC in cassava roots; suggesting that one of these two methods or both may not be accurate. Nevertheless, high correlation coefficients were noted among the methods investigated; suggesting that these methods can be reliably used to screen carotenoids in earlier stages of cassava breeding. Indeed, in the initial stages of breeding, a method that has high repeatability precision can be used in germplasm selection even though its accuracy could be

wanting. The choice of which to use will be largely determined by associated costs, required speed and breeding objectives.

CONCLUSION

This study gave a general overview of the phenotyping methods available across cassava breeding programs. The phenotyping methods were found to have good repeatability, a characteristic that is directly associated with high precision and inversely associated with random errors. Similarly, these methods had high correlations among each other. On this premise, these methods can reliably be used for germplasm selection/screening in the initial stages of breeding. Despite having good repeatability precision, iCheck and Vis/NIR methods appeared to have incongruence in their accuracies. It was recommended that further studies should be done by including a standard method like spectrophotometer to assess their accuracies and identify the more accurate method that can be used at all stages of cassava breeding.

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AUTHOR'S CONTRIBUTION

Chiedozie Egesi and Wilfred Abincha conceptualized, designed the research, collected and analyzed data and drafted the manuscript. Robert Kawuki, Betty E. Owor, Justus Anyieni and Yasmin Hussin supervised the whole process of research and provided technical advice. Ugochukwu Ikeogu developed the Vis/NIRS prediction model used in this work and also contributed in additional concepts and drafting the manuscript. Peter Kulakow, Ismail Rabbi and Elizabeth Parkes assisted in laboratory analyses of carotenoids and supervised the research. The manuscript was reviewed by all authors.

Competing Interest Statement

The authors declare that there are no any competing interests.

Ethical Approval

This article does not contain any studies with live vertebrates or higher invertebrates, humans or human sample performed by any of the authors.

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